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Divulging the anti-acetylcholinesterase activity of *Colletotrichum lentis* strain KU1 extract as sustainable AChE active site inhibitors

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Abstract

Alzheimer's disease (AD), also called senile dementia is a neurodegenerative disease seen commonly in the elderly and is characterised by the formation of β-amyloid plaques and neurofibrillary tangles (NFT). Though a complete understanding of the disease is lacking, recent studies showed the role of the enzyme acetylcholinesterase (AChE) in pathogenesis. Finding new lead compounds from natural sources has always been a quest for researchers. Endophytic fungi are a set of microbes that reside within plants without causing any harm**.** This study focuses on screening endophytes for the production of active acetylcholinesterase inhibitors. Five endophytic fungi were isolated from *Catharanthus roseus* and screened for AChE inhibitory activity. Three isolates were found to inhibit AChE inhibitory activity and were distinguished based on molecular and microscopic methods. The mycelial extract was taken for the bioassay-guided column chromatography and TLC was performed on the active fraction. The GC-MS and NMR analysis identified the active compounds in the extract as 9-hexadecen-1-ol and erucamide. Molecular docking studies revealed that the compounds are thermodynamically feasible and have significant glide scores. Computational studies revealed that the hydroxyl group of 9-hexadecen-1-ol forms a hydrogen bond with Ser 293 in the active site of AChE, whereas the active site interactions were predominantly hydrophobic in the case of erucamide and are reflected in AChE inhibition assays.

Keywords: Acetylcholinesterase inhibitor, Endophytic fungi, *Colletotrichum lentis* strain KU1, *In silico*

Introduction

Alzheimer's disease (AD) is a neurodegenerative condition marked by the gradual and inevitable depletion of neurons in particular brain regions, predominantly the hippocampus (Zhu et al. 2004). Some predominating symptoms include memory loss, language deterioration, poor judgment, and impaired visuo-spatial skills, which sometimes vary with persons (Selkoe and Peter J Lansbury 1999). Age is considered one of the most important predisposing factors of this disorder, affecting over 20 million individuals worldwide. The cost of care associated with the disease is high and is probably beyond most developing countries capability. The pathology of Alzheimer's is a result of the deficiency of the neurotransmitter acetylcholine (ACh) (Čolović et al. 2013). This molecule performs an essential role in transmitting nerve impulses from neuron to neuron, thereby providing smooth nerve activities. Besides the ACh deficiency, numerous other issues are associated with disease progression. Amyloid beta (Aβ) protein aggregation, Neurofibrillary tangles, etc., are a few among them, but whether it is a cause or a symptom is still unclear (Murphy and LeVine 2010; Ferreira-Vieira et al. 2016).

Inhibition of cholinesterase is considered one of the best pharmacotherapeutic methods for treating cognitive symptoms of AD (Lima et al. 2018). This will facilitate acetylcholine levels at the synapses, thus improving neurotransmission (Hornick et al. 2011). Drugs like tacrine, donepezil, rivastigmine, and galantamine are presently used to treat dementia, but they can be replaced with new drugs with reduced side effects. Researchers are interested in finding natural compounds with more bioavailability and reduced side effects as therapeutic agents (Mehta et al. 2012).

Microorganisms are beneficial, mainly due to their ability to produce valuable compounds/molecules called secondary metabolites (Mazzoli et al. 2017). Most drugs, especially antibiotics available presently, are of microbial origin. Besides producing medically

relevant compounds, they are beneficial in many ways. Production of chemicals like ethanol, butanol, vitamins, and usage in pollution control, metal degradation, and oil spills are some of their notable roles (Singh et al. 2016). Their applications are not limited to the field mentioned above, but our interest is in finding certain medically relevant compounds for therapeutic purposes. As mentioned previously, AChE inhibitors can increase the duration of Ach in the synapse and thereby enhance neural functions (Taylor et al. 2009). Our approach is to find valuable compounds from nature, purify them, and analyse their effects *in silico* and *in vitro*. Endophytes belong to a class of curious and interesting microorganisms that form association with plants for competence, survival and reproducing intercellularly (Abdel-Azeem et al. 2019; Gouda et al. 2016). Medicinal plants in their natural environments are ideal hosts for endophytic fungi that may generate bioactive secondary metabolites with medicinal use. These microorganisms reside in the tissue of higher plants without any harm and are promising sources for natural metabolites having biological activities. This study focuses on the Colletotrichum lentis strain's ability to produce compounds that inhibit AChE.

Materials and Methods

Collection of plant material

The already authenticated plant (*Catharanthus roseus*) was collected from Kannur University Campus, Palayad, Kerala (11°47'46.9"N 75°28'03.7"E) and morphologically examined.

Isolation and identification of endophytic fungi

The surface disinfection of the plant sample was done by previously identified method. (Wang et al. 2015). The fresh plant parts of *C. roseus* were given an initial wash with water followed by 70% ethanol treatment by soaking it for 4 min duration. Then, soaked plant parts were washed with sterile water five times and placed in 1% sodium hypochlorite solution for 5 min, followed by washing with distilled water five times. The surface sterilised plant cuttings were

transferred into Potato Dextrose Agar (PDA) and maintained at room temperature. After two weeks of incubation, the mycelial tips were inoculated on clean PDA plates for screening. This method was repeated more than five times for acquiring pure lines, and the mycelia received were saved on a PDA slant. The endophytic fungal species were identified based on culture features, the morphology of fruiting bodies, and spores. The identification was made using standard taxonomic identification manuals (Benjamin 1955). Macroscopic characterisation based on colony morphology was done by growing the isolates in potato dextrose agar (PDA) and observing the colony's size, growth and colour. The cultures that did not sporulate were recorded as sterile.

Fermentation and extraction of metabolites

Fresh mycelia of selected endophytes were transferred to plates of fresh potato dextrose agar (PDA) and allowed to grow at 25°C±2°C for 7-14 days. Colonised PDA plugs (5 mm in diameter) were inoculated to a 250 mL Erlenmeyer flask containing 100 mL Czapex Dox Broth (CDB) medium. (sucrose, 3.0 g; $NaNO_3$, 0.3 g; K_2HPO_4, 0.1 g; yeast extract, 0.1 g; KCl, 0.05 g; $MgSO_4.7H_2O$, 0.05 g; $FeSO_4$, 0.001 g; double-distilled H_2O, 100 mL; autoclaved at 121°C for 20 min). The flasks were placed on an orbital shaking incubator at 120 rpm for 21 days at 25°C ±1°C . The culture broth was filtered through Whatman No. 1 filter paper (pore size: 11 µm). At room temperature, 100 mL of culture broth was extracted thrice with an equivalent amount of chloroform (1:1). To obtain the residual dry extract (crude extract), the fraction was evaporated using a rotary vacuum evaporator. Chloroform is used for selectively extracting low-molecular-weight phenolic compounds and high molecular weight polyphenols (Scholz and Rimpler 1989), having antioxidant activity (Yashavantha Rao et al. 2015). In a study by Arora and Chandra; 2011, they identified chloroform as the best solvent to elute compounds having antioxidant activity than solvents like butanol, chloroform, and ethyl acetate. Hence, chloroform was selected for the extraction in this study.

Determination of Anti-acetyl cholinesterase activity

Ellman's method with slight modification was used to determine Anti-AChE activity (Ellman et al. 1961). The substrate ACh-Iodide and AChE enzyme from Electric-eel were purchased from Sigma Aldrich. 5,5- Dithiobis-2-nitrobenzoic acid (DTNB, Sigma) was used to determine AChE inhibitory assay. The reaction mixture consisted of 10 µL of sample solution dissolved in ethanol, 120 µL phosphate buffer, 50 µL of AChE enzyme (0.4 U/mL), 20 µL of 7.5 µM DTNB, and was incubated for 30 minutes at 37°C temperature. After incubation, 50 µL of 7.5 µM substrate ATCI (acetylthiocholine iodide) was added. OD was measured at 412 nm every 30 seconds for a period of 300 sec. Donepezil, a known AChE inhibitor, was used as a positive control (0.005 mg/ml) to compare the inhibitory activity. 0.1 mg/ml of the extracts were used to test the inhibitory activity. The The time-dependent activity of the enzyme was recorded and represented graphically. The percentage inhibition of AChE activity was measured using the following equation:

$$\text{Inhibition } (\%) = (1 - \text{B/A}) \times 100$$

Where, A = change in optical density of control, and B = change in optical density of test solution.

Molecular characterisation of active strain

The strains were grown in PD broth at 28°C in a rotary incubator at 150 rpm. The morphological and molecular analysis of the selected strain was done using microscopic and ITS gene sequencing. Universal primers ITS1 (5′- TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used to amplify the gene sequence of fungal strain. The conditions for PCR amplification were as follows: initial denaturation at 94°C for 300s; 34 cycles of 60s at 94°C; 45s at 53°C; and 90s at 72°C; and final extension of

600s at 72°C. 1% agarose gel electrophoresis was used to analyse the PCR products. The sequencing reaction was done in a PCR thermal cycler using the BigDye Terminator v3.1 Cycle sequencing Kit following manufacturer protocol. DNA sequences collected from active strains were matched with the MEGA 10.0 program by CLUSTALW (Kumar et al. 2018). The nucleotide BLAST was used to analyse assembled DNA sequence data and was compared with the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/). The multiple alignments were achieved using CLUSTALW and Neighbour-joining analyses, and phylogenetic tree construction was done using MEGA 10(Hall 2013).

Preliminary qualitative biochemical screening of fungal metabolites produced by endophytic fungi

The fungal crude extract was analysed for secondary metabolites like alkaloids, flavonoids, phenolics, saponins, steroids, tannins, and terpenoids. Each extract was chemically tested to confirm the presence of particular secondary metabolites according to standard procedures (Seigler 1998). The presence or absence of any secondary metabolite was confirmed by a visible colour change or precipitate formation.

Purification of compounds showing acetylcholinesterase inhibitory activity

Bioassay-guided fractionation of cholinesterase inhibitor was carried out using column chromatography and Thin Layer Chromatography techniques (TLC) (Joseph and Mahapatra 2018). Column chromatography of the crude extract was conducted by the wet packing method in hexane using silica gel (Mesh 60-120). The column was run using hexane, chloroform, and methanol by gradient elution technique (Ayyolath et al. 2020). The silica gel column was eluted with 50 mL of each solvent. Each fraction was analysed for acetylcholinesterase inhibitory activity. A total of 25 eluates (hexane: chloroform) were collected, of which nine fractions showed positive results. Further purification was carried out using silica gel of mesh size 230-400 using hexane: toluene (with the increase in polarity of 10) as the mobile phase. Similar

fractions were combined after TLC analysis. The fractions were evaporated to dryness, and the residues were dissolved in methanol.

The preparative thin layer chromatography analysis was carried out using the pooled fractions obtained from column chromatography. The separation was carried out on 20 cm × 20 cm glass plates coated with silica gel-G with toluene: ethyl acetate (6:4 % v/v) as the mobile phase. (Ayyolath et al. 2020) The disclosed bands were then scraped off, dissolved in methanol, and filtered. The solvent was removed by evaporation. The sample was tested for cholinesterase inhibition, and GC-MS followed by NMR analysis was conducted to identify the active compounds.

Identification of active compounds

GC-MS analysis was carried out to characterise the fungal pigment. ^{1}H NMR and ^{13}C NMR are employed to confirm the compound's structure further. The GC-MS analysis of the active fraction was performed using Thermo Scientific Trace 1300 Gas chromatograph equipped with ISQ-QD Mass spectrometer with TG-5 MS column (30 m × 0.25 mm; 0.25 μm). Helium gas (99.99%) was used as the carrier gas at a constant flow rate of 1 mL per min, and an injection volume of 1µL was employed (Split ratio 1:8). An injection port temperature of 280°C and an ion-source temperature of 200°C were set. The oven temperature was programmed from 70°C for 2 min with an increase of 6°C per min to 260°C with a hold time of 2 minutes: oven temperature and GC running time were adjusted accordingly in each extraction. Total GC running time was 29.1 minutes. The components in the active fraction were identified based on the mass spectra of NIST library data. ^{1}H NMR and ^{13}C NMR spectra of the compounds were documented on Shimadzu AMX 400-Bruker, 400 MHz spectrometers using DMSO-d6 as solvent.

Molecular Docking; Protein and ligand Structural refinement

The crystal structure of protein AChE (PDB ID: 4EY7) was selected from the protein data bank (PDB) and used to model protein structure in the study. Schrödinger Maestro 10.4v has been used for protein preparation. (Sastry et al. 2013). The files were downloaded and saved as 4EY7.pdb. The protein preparation wizard of Schrödinger protein structure was processed for its bond orders, alleviating potential steric clashes, formal charges, assigning disulfide bonds, missing hydrogen atoms, and incomplete and terminal amide groups. Water molecules that were present 5A^{o} beyond heteroatoms were removed. Energy minimisation was performed using the default constraint of 0.3 A° RMSD and OPLS_2005 force field. PLIP (protein-ligand identifier profiler) webserver was used to identify the binding pockets and amino acid residues and generate a receptor Grid using GLIDE. (Park et al. 2009)

The Ligprep module was used to prepare the ligand structures retrieved from PubChem Database (Parasuraman et al. 2012). Ligands downloaded in the 2D structure were converted to 3D, considering all the parameters. LigPrep generates the least 3D energy structure with the proper chirality. Hydrogen was inserted, angles and bond length was standardised, and the probable tautomer was introduced at a pH of 7.0. The final step of LigPrep includes energy minimisation of ligand structures using the OPLS_2005 force field (Tripathi et al. 2013).

Induced-Fit Molecular Docking

The molecular docking study was performed using the Schrödinger Maestro v.10.4 GLIDE module (Friesner et al., 2006). Glide docking addresses the concept of a rigid receptor even if Van der Waals radii of non-polar atoms are scaled, which excludes near-contact constraints, and can be used to model a minor "give" in the receptor and ligand. Previously, the prepared receptor grid and ligand were used for docking, and Extra-precision (XP) docking was adopted using the OPLS_2005 force field.

ADME property, target prediction analysis, and toxicity* e*valuation

Determining the lead compound's ADME (Absorption, Distribution, Metabolism, and Excretion) properties is an important criterion. The Swiss ADME (http:/www.swissadme.ch/), a web-based tool, was used to determine ADME properties. Lipinski's rule of five, the solubility of the drug, drug likeliness, and pharmacokinetic properties were the predominant parameters taken into consideration in this study (Daina et al. 2017).

In silico toxicity, determination helps predict the nature of the drug in living systems. These methods rely on learning the relation between chemical structure and its nature, and consider that compounds with similar structures behave similarly. pkCSM web server (Pires et al. 2015) was used for the toxicity assessment through http://structure.bioc.cam.ac.uk/pkcsm.

Evaluation of AChE inhibitory activity of the selected compounds

Based on the GCMS analyses prediction, compounds with the highest probability were selected for further studies. The chosen compounds 9-Hexadecen-1-ol and Erucamide were evaluated for *in vitro* AChE inhibitory activity. The assay used the AChE from electric eel and the substrate ATCI. The compounds were dissolved in ethanol and used at 1 μM.

Results

Isolation of endophytic fungi

Cultures with characteristics morphology of fungi have been chosen for further studies (**Figure 1A**). Endophytic fungi and endophytic actinomycetes were isolated from asymptomatic stem tissues of *C. roseus*. In addition, the final rinse water did not exhibit any microbial growth even after 10-20 days of incubation at 25ºC±2ºC, showing that the surface sterilisation procedure was effective in killing epiphytes, and the subsequent isolates were considered as true endophytes.

Morphological identification and diversity of endophytic fungi

Preliminary identification of endophytic fungi was carried out based on morphological characteristics. The fungal isolates were then identified at least up to genus level and identified as *Curvularia sp*., *Geotrichum sp*., *Gliocladium sp*., *Fusarium sp*., and *Colletotrichum sp*.

Acetylcholinesterase inhibition assay

In this assay, acetylthiocholine is used as the substrate, and hydrolysis of the substrate is measured by analysing the formation of the yellow 5-thio-2-nitrobenzoate anion, due to the reaction of thiocholine with 5,5'–dithio-bis-2-nitrobenzoic acid. Inhibitors of AChE are used in many disorders such as Alzheimer's disease, and myasthenia gravis. The study noticed a remarkable inhibition for the chloroform extract of *Colletotrichum sp*. (53 %). The *Gliocladium sp.* and Fusarium *sp.* do not show any activity. The percentage of inhibition for *Curvularia sp*. and *Geotrichum sp*. were 46.52% and 24.45%, respectively, while standard inhibitor Donepezil shows 81.09% inhibition **(Fig 1B)**.

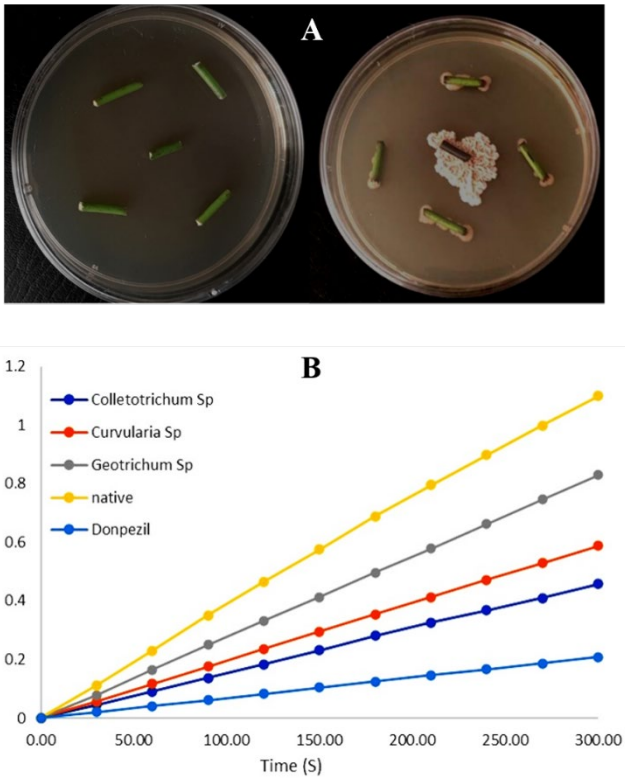

Fig 1: A) Figure showing the isolation of endophytic fungi from stem tissues. B) Anti acetylcholinesterase inhibitory activity of fungal extracts

Molecular identification of selected fungi

The ITS region coding sequence of the strain has been amplified using the Polymerase chain reaction. The amplified product was sequenced, and the sequence obtained was compared to the sequence from the National Biotechnology Information Centre's (NCBI) Nucleotide Database. The selected fungal strain was grown in potato dextrose liquid medium, harvested by filtration, washed with distilled water, and rapidly frozen in liquid nitrogen. DNA isolation was carried out using NucleoSpin® Plant II Kit (Macherey-Nagel), following the manufacturer's guidelines. Genomic DNA of fungi was isolated, as observed in (**Fig 2A),** and the ITS region was amplified from the DNA of the isolated fungus. Amplified products were run on 1.2% (w/v) agarose gel, and the amplicon was found to be of ~1 kb, as observed in **Fig 2A(a)**. BLASTn showed a 100 per cent resemblance to *Colletotrichum lentis* strain CBS 127604 small subunit ribosomal RNA gene, partial sequence. This is a partial sequence of 576 bp comprising small subunit ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and large subunit ribosomal RNA. The sequence showed the highest similarity with *Colletotrichum lentis*, a member of *Glomerellaceae* family. Based on the morphological and molecular conformations, the isolate KU1 was identified as *C. lentis* and submitted under NCBI accession **MW055916.1**. The evolutionary history was interpreted using the neighbour-Joining method. (Saitou and Nei 1987) **(Fig 2B)**. The ideal tree with the sum of the length of the branch = 0.25261213 is shown. The confidence chance (multiplied by 100) that the length of the inner branch is greater than 0, as estimated by the bootstrap test.

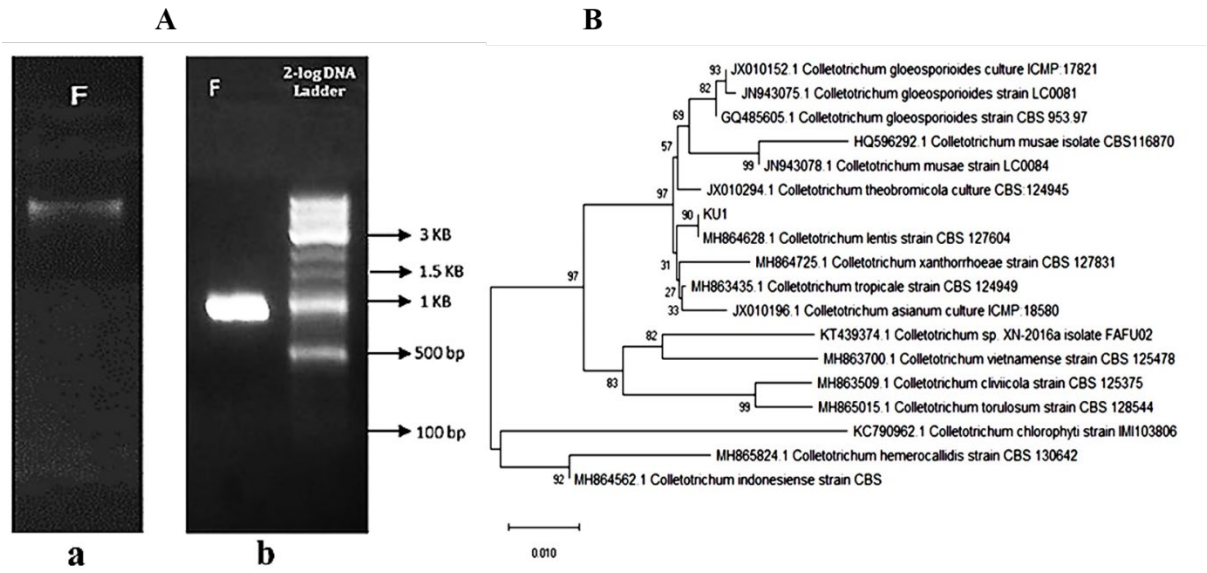

Fig 2: A) Electrophoresed Fungal genomic DNA (a) and PCR product of amplified genomic DNA (b). B) Phylogenetic tree generated using Mega X.

Biochemical screening

Based on the acetylcholinesterase inhibition assay of the fungal extracts, the preliminary biochemical screening was carried out for the chloroform extract of *C. lentis*. The biochemical analysis gives a general idea regarding the nature of the chemical constituents of the samples. The chloroform extract of *C. lentis* showed the presence of alkaloids, flavonoids, terpenoids, and steroids.

Isolation of compounds showing acetylcholinesterase inhibitory activity

Isolation of compounds from *C. lentis* chloroform extract showing protease inhibitory activity was carried out by column chromatography using silica gel of different mesh sizes. The fractions were eluted using different solvent systems ranging from Hexane → Chloroform → Methanol with an increase in polarity. The fractions were evaluated for protease inhibition using a spectrophotometric time scan assay. Fractions showing a similar banding pattern in TLC analysis were pooled together and subjected to preparative TLC **(Fig 3A)**. The compounds

in the active fraction obtained after preparative TLC was analysed by UV-Visible spectrophotometry, GC MS and NMR analysis.

3.7 Spectrophotometric analysis of the active fraction

The GC-MS analysis of the active fraction of *C. lentis* indicated the presence of 12 major compounds of which 9-Hexadecen-1-ol and Erucamide are the major constituents (**Table 1**). The details of the phytoconstituents are given in the table along with the chromatogram **(Fig 3B)**. The ^{1}H NMR and ^{13}C NMR analysis of the fractions confirms the structural corelation with 9-Hexadecen-1-ol and Erucamide. The NMR data cordinates for 9-Hexadecen-1-ol is ^{1}H NMR: δ 0.87 (3H, t, *J* = 7.0 Hz), 1.18-1.49 (18H, 1.24 (quint, *J* = 7.0 Hz), 1.24 (quint, *J* = 7.0 Hz), 1.24 (quint, *J* = 7.0 Hz), 1.25 (quint, *J* = 7.0 Hz), 1.28 (h, *J* = 7.0 Hz), 1.28 (quint, *J* = 7.0 Hz), 1.37 (tt, *J* = 7.1, 7.0 Hz), 1.42 (tt, *J* = 7.4, 7.0 Hz), 1.43 (tt, *J* = 7.4, 7.0 Hz)), 1.73 (2H, tt, *J* = 7.2, 7.0 Hz), 1.89-2.02 (4H, 1.95 (q, *J* = 7.4 Hz), 1.97 (q, *J* = 7.4 Hz)), 3.33 (2H, t, *J* = 7.2 Hz), 5.29-5.45 (2H, 5.37 (dt, *J* = 15.6, 7.4 Hz), 5.37 (dt, *J* = 15.6, 7.4 Hz)). ^{13}C NMR: δ 14.0 (1C, s), 22.6 (1C, s), 25.8 (1C, s), 29.1-29.2 (2C, 29.1 (s), 29.1 (s)), 29.3-29.4 (4C, 29.4 (s), 29.4 (s), 29.4 (s), 29.4 (s)), 31.8 (1C, s), 32.5-32.5 (2C, 32.5 (s), 32.5 (s)), 32.6 (1C, s), 62.2 (1C, s), 132.5-132.7 (2C, 132.6 (s), 132.6 (s)). The NMR data cordinates obtained for Erucamide is ^{1}H NMR: δ 0.86 (3H, t, *J* = 7.0 Hz), 1.16-1.34 (24H, 1.23 (tt, *J* = 7.0, 6.9 Hz), 1.23 (quint, *J* = 7.0 Hz), 1.23 (quint, *J* = 7.0 Hz), 1.23 (quint, *J* = 7.0 Hz), 1.23 (quint, *J* = 7.0 Hz), 1.23 (quint, *J* = 7.0 Hz), 1.24 (tt, *J* = 7.0, 6.9 Hz), 1.24 (quint, *J* = 7.0 Hz), 1.24 (quint, *J* = 7.0 Hz), 1.26 (quint, *J* = 7.0 Hz), 1.27 (tt, *J* = 7.7, 7.0 Hz), 1.28 (h, *J* = 7.0 Hz)), 1.36-1.59 (6H, 1.43 (tt, *J* = 7.4, 7.0 Hz), 1.43 (tt, *J* = 7.4, 7.0 Hz), 1.52 (tt, *J* = 7.7, 7.4 Hz)), 1.89-2.03 (4H, 1.95 (q, *J* = 7.4 Hz), 1.97 (q, *J* = 7.4 Hz)), 2.19 (2H, t, *J* = 7.4 Hz), 5.26-5.41 (2H, 5.33 (dt, *J* = 11.0, 7.4 Hz), 5.33 (dt, *J* = 11.0, 7.4 Hz)). ^{13}C NMR: δ 14.0 (1C, s), 22.6 (1C, s), 25.4 (1C, s), 27.1-27.3 (2C, 27.2 (s), 27.2 (s)), 29.1-29.2 (2C, 29.1 (s), 29.1 (s)), 29.3-29.4 (10C, 29.4 (s),

29.4 (s), 29.4 (s), 29.4 (s), 29.4 (s), 29.4 (s), 29.4 (s), 29.4 (s), 29.4 (s), 29.4 (s)), 31.8 (1C, s), 34.9 (1C, s), 130.3-130.5 (2C, 130.4 (s), 130.4 (s)), 174.0 (1C, s). (**Fig 4**)

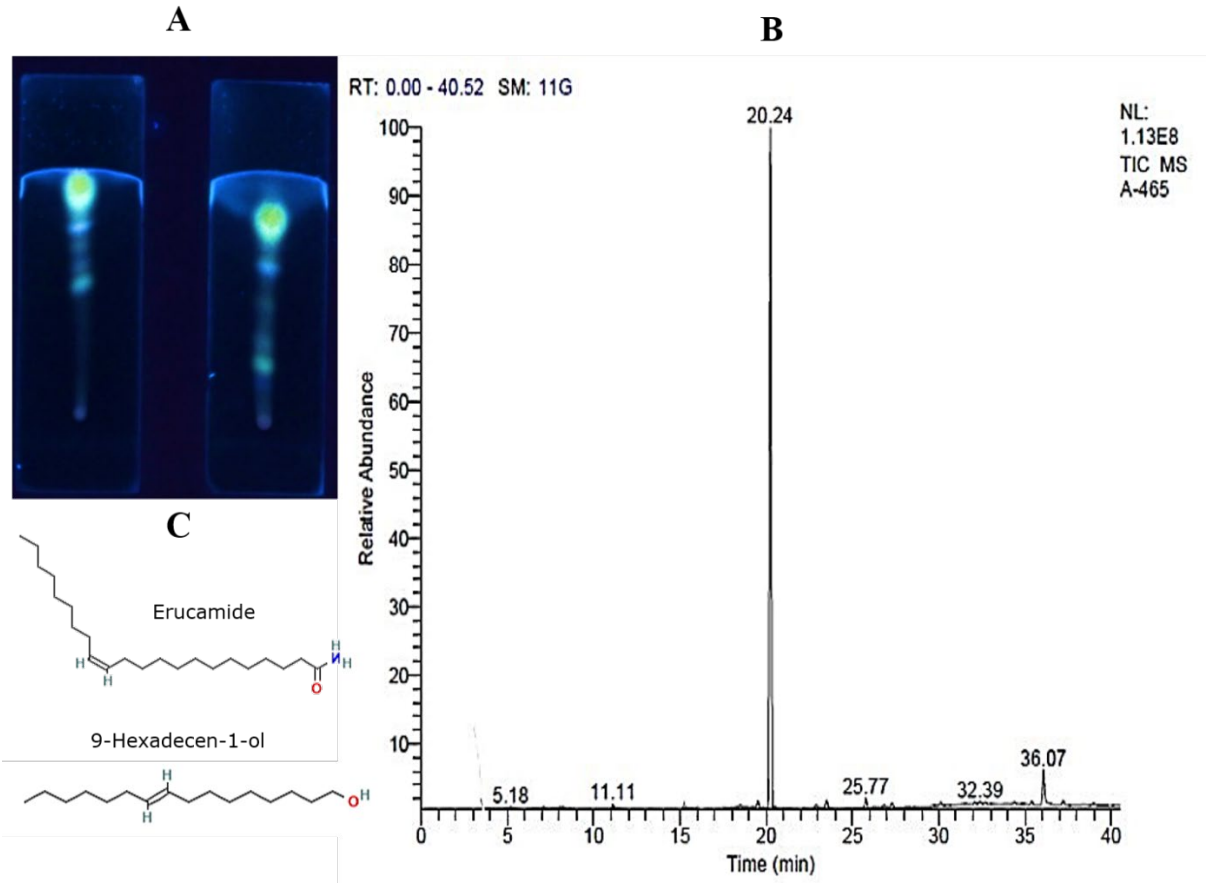

Fig 3: A) TLC image of the active fraction at 366 nm. B) GC MS chromatogram. C) Structure of 9-Hexadecen-1-ol and Erucamide.

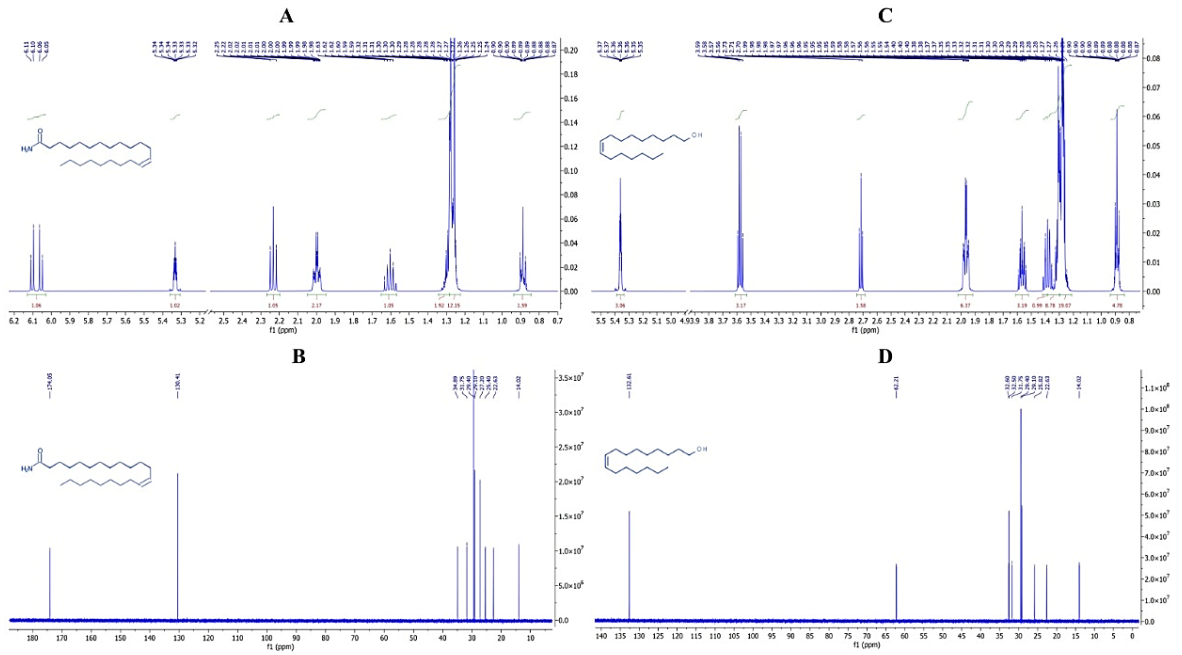

Fig 4: NMR spectra of the active compounds. A,C) ^{1}H NMR spectra of -Hexadecen-1-ol and Erucamide. B,D) ^{13}C NMR spectra of of -Hexadecen-1-ol and Erucamide.

Molecular Docking studies of the selected compounds Acetylcholinesterase

Molecular docking studies were carried out with the 12 compounds identified by GC-MS analysis using the crystal structure of AChE. The results selected two compounds, 9-Hexadecen-1-ol and Erucamide, with significant glide scores **(Fig 3C)**. The PubChem Data Base and Protein Data Bank were used to retrieve the crystal structures of Cholinesterase (PDB: 4EY7), 9-Hexadecen-1-ol, Erucamide, and Donepezil (PubChem CID: 5283282, 5365371, 3152). All the water molecules were removed from the complex for protein preparation, and polar hydrogen was added. The disulfide bonds were properly treated. For protein structure optimisation and minimisation, the OPLS-2005 force field was used. The minimisation mechanism of the prepared protein structure is terminated when an RMSD of 0.30 Å is exceeded. Using force field OPLS-2005, potential conformations or orientations were produced at a pH of 7.0±2.0. The best pose was chosen based on the glide ranking. The XP glide docking analysis of 9-Hexadecen-1-ol, Erucamide, and Donepezil with AChE reveals

that the compounds predominantly interact at the active site (Hydrophobic pocket) **(Fig 5 A)** through Van der Wall's forces. All of the proposed compounds interact at the hydrophobic pocket of the receptor through the Van der wall's forces of interaction. The compound 9-Hexadecen-1-ol interacts with active site residues such as TRP286, TYR337, PHE338, and TYR341 with a significant binding energy of 54.962 Kcal/mol. Erucamide interacts with the active site reduces such as TRP286, LEU289, PHE338, TYR341, ASP74, and TYR337 and has the binding energy of -63.46776 Kcal/mol. The standard target Donepezil interacts with AChE through amino acid residues such as TYR341, TRP286, and TYR124 with a significant binding energy of -29.047 Kcal/mol **(Fig 5B)**. Using the MMGBSA method, binding energies have been determined, and the different components that contribute to the total binding energy are given **(Table 2)**.

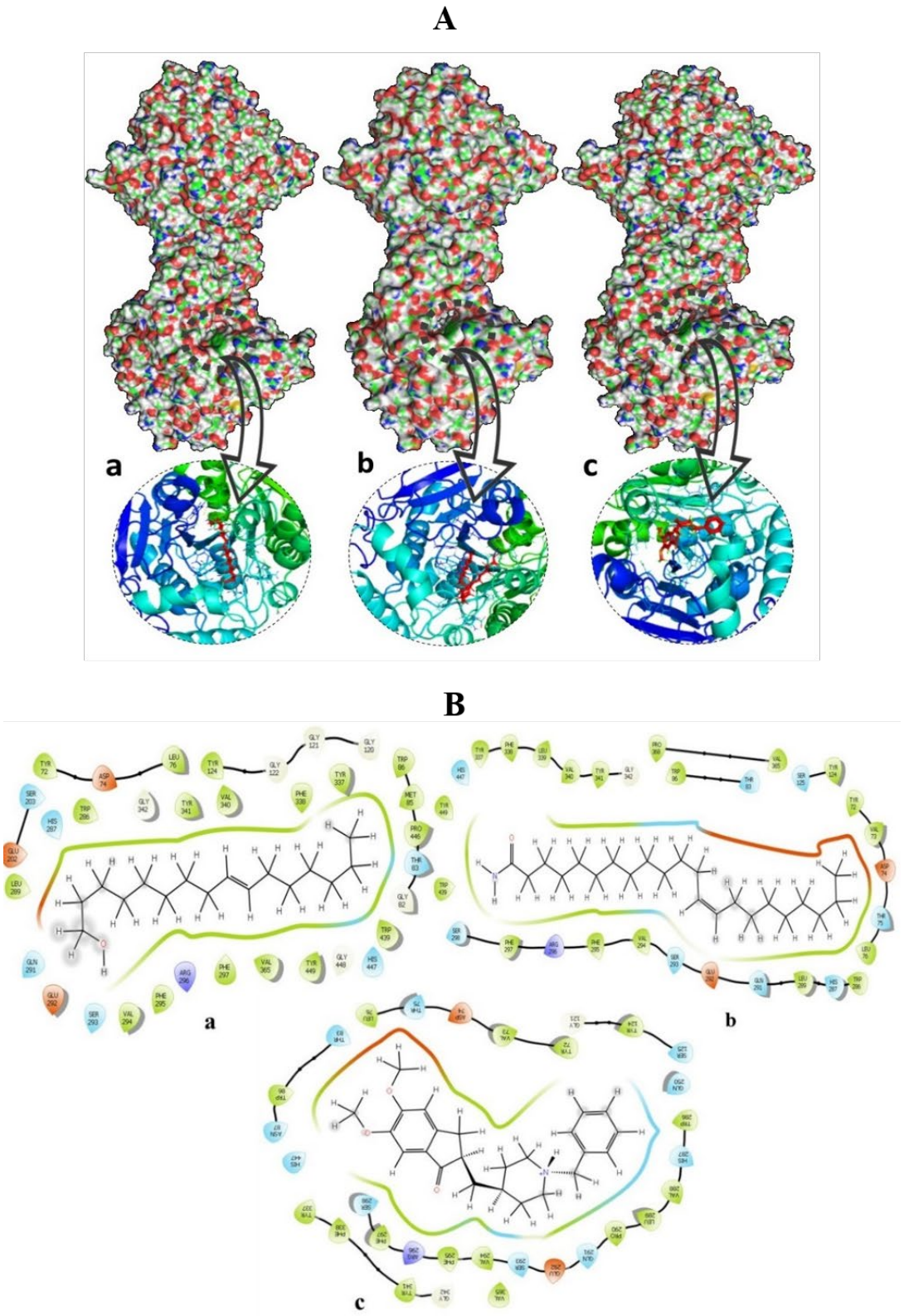

Fig 5: A) The binding of (a) 9-Hexadecen-1-ol (b) Erucamide (C) Donepezil at the active site of Acetylcholinesterase. B) Binding mode of (a) 9-Hexadecen-1-ol (b) Erucamide (c) Donepezil at the active site of Acetylcholinesterase.

ADME analysis and Target prediction

The ADME analysis of 9-Hexadecen-1-ol and Erucamide obeys the drug-likeness rule like the Lipinski rule of five (MW ≤ 500, MLOGP ≤ 4.15, N or O ≤ 10, NH or OH ≤ 5) with one violation (MLOGP>4.15) **(Fig 6 A)**. The other calculated ADME parameters for 9-Hexadecen-1-ol are lipophilicity: 6.63, Size: 240.42 g/mol, Polarity: 20.23Å, insolubility: 5.39e-03 mg/ml; 2.24e-05 mol/l, insaturation: 0.88, flexibility: 13 **(Fig 6A(a))**. The ADME parameters for Erucamide are lipophilicity: 9.16, Size: 337.58g/mol, Polarity: 43.09 Å², insolubility: 1.20e-04 mg/ml; 3.55e-07 mol/l, insaturation: 0.86, flexibility: 19 (**Fig 6A(b)**). Target prediction using Swiss Target Prediction allowed finding other possible targets for 9-Hexadecen-1-ol and Erucamide **(Fig 6B)**. The output data revealed that the nuclear receptors and enzymes are the main target class for 9-Hexadecen-1-ol. The analysis of Erucamide shows enzymes and oxidoreductases as predicted targets.

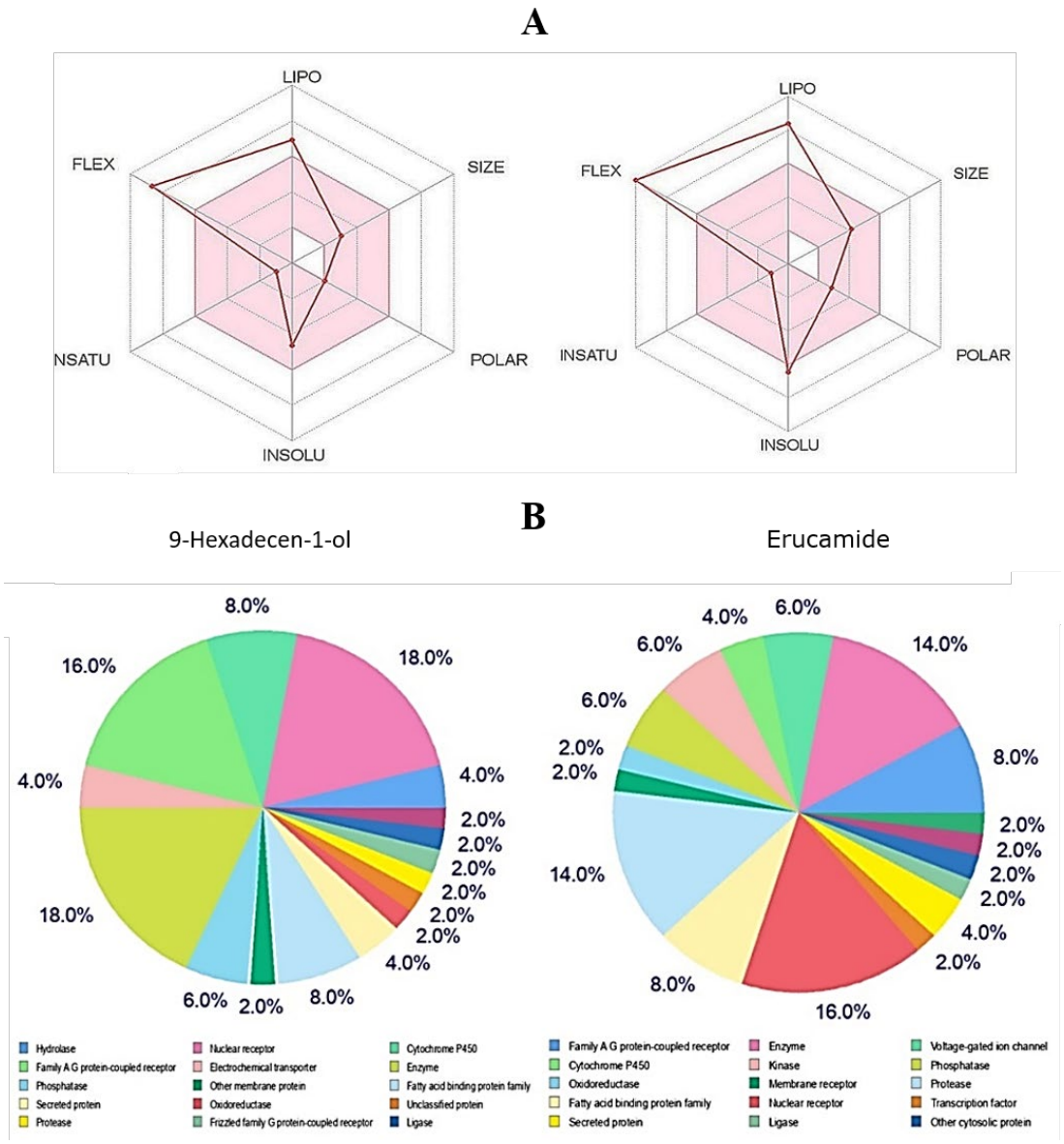

Fig 6: A) The ADME profile of 9-Hexadecen-1-ol and Erucamide. B) Predicted target for 9-Hexadecen-1-ol and Erucamide.

Evaluation of toxicity

The 9-Hexadecen-1-ol toxicity evaluation suggests that the drug has no AMES toxicity. The maximum human tolerance dosage of 9-Hexadecen-1-ol is 0.026 mg/kg/day, and the acute oral toxicity of rats (LD_{50}) is approximately 1.55 mol/kg, with an estimated oral chronic toxicity of rat (LOAEL) of 1.156 mg/kg b.w. / day. Hepatotoxicity and skin sensitisation are not found in the drug. The compound is not an inhibitor of hERG I or II and has *T. pyriformis* Toxicity

(2.129 log ug/L) and Minnow Toxicity (0.802 log µM). In humans, the median tolerance dosage for Erucamide was 0.469 mg/kg/day. Erucamide's oral acute toxicity (LD_{50}) and Oral Chronic Toxicity (LOAEL) for rats are 1.873 mol / kg and 0.796 mg / kg b.w. / day. Erucamide is not an hERG-I inhibitor and has hERG-II inhibition. The Minnow and *T. pyriformis* toxicity of Erucamide were 2.084 µM and 0.665 ug/L, respectively.

Evaluation of cholinesterase inhibitory activity by the selected compounds

The selected compounds, 9-Hexadecen-1-ol and Erucamide, were evaluated for in vitro AChE inhibitory activity (**Fig 7**). The assay was carried out using AChE from electric eel and the substrate ATCI following the spectrophotometric time scan assay for 300s at 412 nm. The selected compounds were dissolved in Ethanol and used at a concentration of 1 µM. The percentage of inhibition was calculated to be 78.98% and 86.03% for Erucamide and 9-Hexadecen-1-ol, respectively.

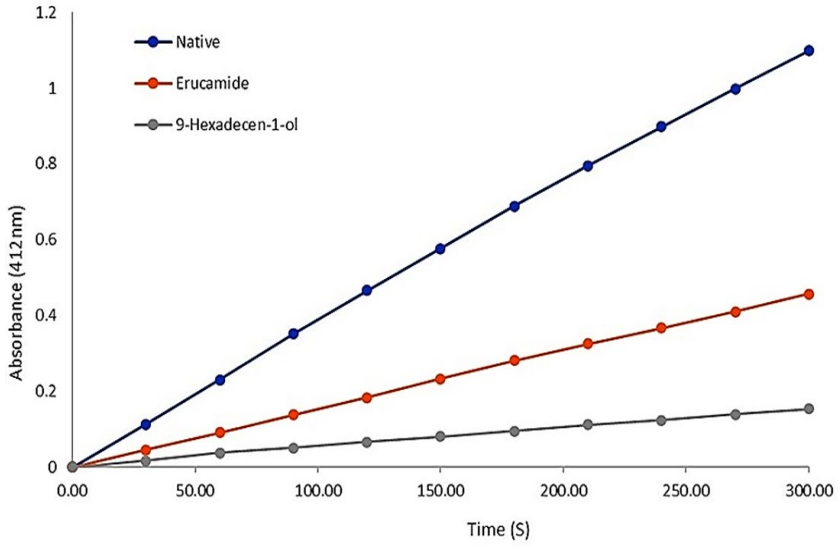

Fig 7: Cholinesterase inhibitory activity of Erucamide and 9-Hexadecen-1-ol.

Discussion

Exploring the potentials of endophytic fungi is of significant interest to researchers. Alzheimer's, a neurodegenerative disease, has no complete cure so far. The use of cholinesterase inhibitors as a pharmacological agent proved to be effective in treating Alzheimer's disease. So, isolation of cholinesterase inhibitors from several endophytic fungi was carried out and later limited to *C. lentis* an endophyte isolated from *C. roseus* based on its ability to produce cholinesterase inhibitors. Molecular identification of fungi was done by ITS sequencing, and the strain was identified and deposited in NCBI under the ID **MW055916.1**. The fungi were mass cultured, and the extract was collected to check its activity. Purification was achieved through techniques like bio-assay guided column chromatography followed by TLC. The GC-MS analysis of the active fraction identified two potential compounds, 9-Hexadecen-1-ol and Erucamide, as major compounds. The structural correlation of the identified compounds was further confirmed by NMR spectroscopic analysis and confirmed the structural backbone of the compounds. Docking studies were carried out, and these compounds showed high binding energy and good docking scores, and the thermodynamic binding of these at the hydrophobic pocket of the receptor molecule was feasible. The compound 9-Hexadecen-1-ol interacts with active site residues such as TRP286, TYR337, PHE338, and TYR341 with a binding energy of 54.962 Kcal/mol. Erucamide interacts with the active site reduces such as TRP286, LEU289, PHE338, TYR341, ASP74, and TYR337 and has the binding energy of -63.46776 Kcal/mol. The compounds isolated were further checked for other essential parameters like ADME and showed acceptable values, ensuring the drug likeliness of these compounds. Target prediction studies showed that nuclear receptors and enzymes were the main target class for 9-Hexadecen-1-ol. The compounds 9-Hexadecen-1-ol and Erucamide were assayed with cholinesterase and showed inhibition of 78.9% and 86.03%. Toxicity studies did not show acute oral toxicity, *T. pyriform*, toxicity or minnow toxicity. The level of active compound production is low in endophytic fungi. Further studies may be

required for optimisation. Knowing the specific parameters needed for growth can improve strain growth and yield of active secondary metabolites., when endophytic fungi are used as drug sources.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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